

STRONGLY ENHANCED TOXICITY OF THE MUSHROOM TOXIN α -AMANITIN BY AN AMATOXIN-SPECIFIC FAB OR MONOCLONAL ANTIBODY

HEINZ FAULSTICH,¹* KARIN KIRCHNER¹ and MASSIMO DERENZINI²

¹Max-Planck-Institut für Medizinische Forschung, Heidelberg, F.R.G., and

²Istituto di Patologia Generale, Università di Bologna, Italia

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H. FAULSTICH, K. KIRCHNER and M. DERENZINI. Strongly enhanced toxicity of the mushroom toxin α -amanitin by an amatoxin-specific Fab or monoclonal antibody. *Toxicon* 26, 491–499, 1988. — A monoclonal antibody, with high affinity against the mushroom toxin α -amanitin, was prepared. Administration of the Fab fragment of the monoclonal antibody to mice caused a 50-fold increase in α -amanitin toxicity. Electron micrographs showed normal appearance of hepatocytes but typical, amanitin-induced lesions in cells of the proximal convoluted tubules of the kidney. The pronounced nephrotoxicity is mainly explained by glomerular filtration and tubular reabsorption of the Fab-amatoxin complex and, to a lesser extent, of the immunoglobulin–amatoxin complex, which is still c. twice as toxic as free α -amanitin. To our knowledge this is the first reported case where immunoglobulins or their fragments enhance rather than decrease the activity of a toxin. Accordingly, immunotherapy of *Amanita* mushroom poisoning in humans does not appear promising.

INTRODUCTION

EARLY ATTEMPTS at using serum to reverse mushroom toxicity date back to the beginning of this century (DUJARRIC DE LA RIVIÈRE, 1929). At that time a horse was 'immunized' with crude extracts of *Amanita phalloides*, but the efficacy of the serum was never clearly proven. In retrospect it seems unlikely that native amatoxins, peptides of c. 900 mol. wt, would exhibit any immunogenic activity. Antibodies against amatoxins can be obtained when the peptides are conjugated with proteins. However, amatoxins bound to proteins are extremely poisonous for animals (CESSI and FIUME, 1969; DERENZINI *et al.*, 1973; ONETTI *et al.*, 1974). Alpha- and β -amanitin were tolerated, however, when attached to a glycoprotein, fetuin, as a carrier. The sera obtained were mainly used for diagnostic purposes (FAULSTICH *et al.*, 1975, 1982; FIUME *et al.*, 1975; FAULSTICH, 1984).

Rabbit antibodies have been assayed also for their immunotherapeutic efficacy (KIRCHNER and FAULSTICH, 1986). It was shown that a purified fraction of the polyclonal immunoglobulins was of no therapeutic value in mice, but rather increased the toxicity of α -amanitin by a factor of two. In the present study we describe the preparation of a monoclonal antibody against α -amanitin, and an approach to immunotherapy using this immunoglobulin or its Fab fragment.

*Author to whom request for reprints should be addressed.

MATERIALS AND METHODS

[³H]-6-*O*-methyl-dehydroxymethyl- α -amanitin (spec. activity = 7.4 Ci/mmol) was prepared in our laboratory (FAULSTICH *et al.*, 1975). The purity of the labeled compound was ascertained by its typical U.V. spectrum and by thin layer chromatography. On silica (HF²⁴, Merck, Darmstadt) developed with chloroform (65) methanol (25) water (4) it represented a single spot detectable by U.V.-absorption. Scraped off and eluted with methanol the extract contained > 90% of the total radioactivity applied to the silica plate.

Beta-amanitin was prepared from aqueous extracts of *Amanita phalloides* mushrooms by chromatographic procedures (FAULSTICH *et al.*, 1973), performed on a preparative scale. The toxin was coupled to fetuin (Sigma, Munich) as described previously (KIRCHNER and FAULSTICH, 1986). The molecular ratio of amatoxin attached to the protein as determined by spectrophotometry ($\epsilon_{210} = 13,500 \text{ M}^{-1}\text{cm}^{-1}$) was 1.8. Aliquots containing 25 μg amatoxin were mixed with Freund's complete adjuvant and injected intracutaneously into Wistar rats of 200 g body weight. Two booster injections, each containing 30 μg amatoxin in 0.9% NaCl, were administered i.m. 4 and 20 weeks after the first injection. Blood samples (0.3 ml) taken from the tail vein 3 weeks after each antigen injection were analyzed for their titers of amatoxin-specific antibodies by ELISA.

Enzyme-linked immunosorbent assay (ELISA) was performed according to the method of ENGVALL (1980). The assay used an α -amanitin conjugate of bovine serum albumin (FAULSTICH and FIUME, 1985; FAULSTICH *et al.*, 1983) attached to the walls of microtiter wells (Dynatech, Denkendorf, FRG) as immunoadsorbant, and anti-rat-IgG (of the rabbit) coupled to horseradish peroxidase (Miles, Frankfurt) for detection.

Spleen cells of the immunized rats were fused with mouse myeloma cells (P3-X-63-Ag8-653), and hybridoma colonies were isolated according to the procedure of LONAY *et al.* (1981). Colonies producing amatoxin-specific antibodies were sub-cloned by the limiting dilution technique (GOODING, 1980). A selected amatoxin-specific hybridoma clone was raised in nude mice. Ascites fluid was harvested after 10 to 20 days and the monoclonal antibody was purified by affinity chromatography on Protein-A-Sepharose Cl 4B (Pharmacia, Uppsala) (MCGREGOR *et al.*, 1983). The immunoglobulin was characterized by immunodiffusion (OUCHTERLONY, 1968) using isotype-specific antisera (Miles, Frankfurt). Fab-fragments were obtained (ROUSSEAU *et al.*, 1983) by digestion with mercuripapain. The Fab-fragment was separated from Fc-fragment and undigested immunoglobulin by passage through a Protein-A-Sepharose Cl 4B column. Spectrophotometry of the amatoxin-immunoglobulin complex and the equilibrium dialysis experiments have been described elsewhere (KIRCHNER and FAULSTICH, 1986).

Female mice (NMRI) of 18–25 g body-weight were poisoned by i.p. administrations of various doses of α -amanitin. About one min after the toxin injection the animals received i.v. equivalent amounts of monoclonal IgG or Fab. All protein fractions contained 0.9% NaCl and were sterilized by ultrafiltration immediately before use. A group of mice received various doses of α -amanitin i.v. complexed with IgG. The complex was prepared by incubating 1 equivalent of IgG with 2 equivalents of α -amanitin 1 hr before administration to the animals.

For electron microscopy, kidney samples were fixed, immediately after animal sacrifice, in 2.5% glutaraldehyde in 0.1 M Sørensen buffer, pH 7.2 and post-fixed in 1% OsO₄ in the same buffer. After alcohol dehydration, the samples were embedded in Epon. Ultrathin sections were stained with uranium and lead.

RESULTS

Preparation of the monoclonal antibody and its Fab fragment

Ascites fluid of nude mice contained up to 7 mg/ml amatoxin-specific IgG. Because of the absence of host-specific immunoglobulins the antibody was easily purified by affinity chromatography on Protein-A-Sepharose (MCGREGOR *et al.*, 1983). In SDS-gel electrophoresis the purified immunoglobulin appeared as one heavy chain and one light chain (Fig. 1).

The subclass of the monoclonal IgG was determined by immunodiffusion (OUCHTERLONY, 1968), and found to be IgG2a. Elution of an IgG2a from an affinity column required a glycine/HCl buffer of pH 3, and although it was neutralized immediately the treatment may have caused partial denaturation of the globulin. Spectrophotometric examination of the binding capacity (KIRCHNER and FAULSTICH, 1986) showed, however, that the binding ratio of α -amanitin to immunoglobulin was 1.92 : 1, which is close to the expected value of 2 : 1.

For preparation of the Fab fragments the IgG2a was digested with mercuripapain. The reaction was complete after 2–3 hr as shown by the appearance of only two bands in SDS-PAGE corresponding to Fab and Fc fragments. As illustrated in Fig. 2, undigested

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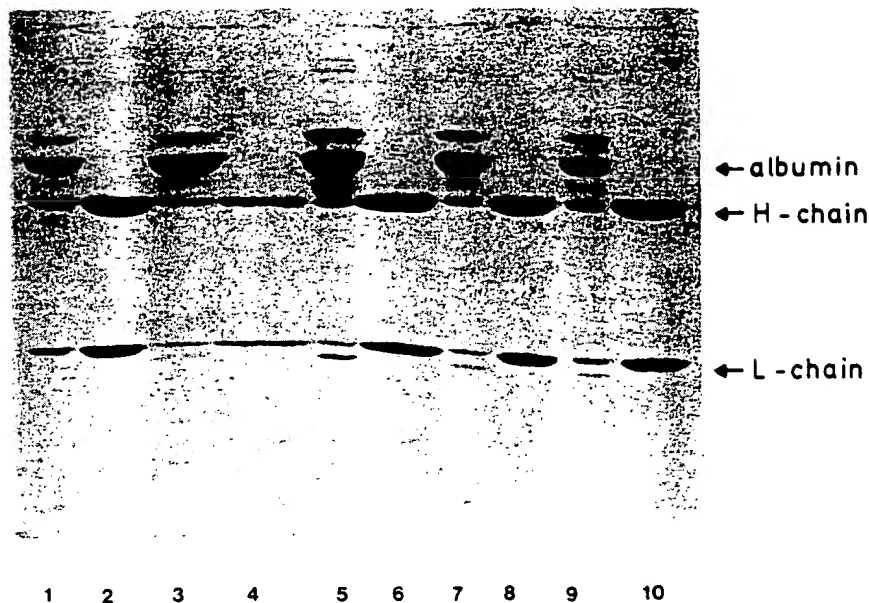


FIG. 1. AFFINITY PURIFICATION OF A MONOCLONAL IMMUNOGLOBULIN FROM THE ASCITES OF NUDE MICE AS FOLLOWED BY SDS-PAGE.

Lane 1, 3, 5, 7, 9: crude ascites of 5 animals (the major band is mouse albumin). Lane 2, 4, 6, 8, 10: the monoclonal immunoglobulin fractions after purification. Only the heavy (H-) and light (L-) chain of the IgG are visible.

heavy chain was still visible after 1 hr, while after 4 hr of digestion side products were detected. Again, Protein-A-Sepharose was used to separate Fc fragments and unmodified IgG from Fab, which was obtained in ca. 90% yield.

The affinity of the monoclonal IgG2a and its Fab fragment for amatoxins was assayed by equilibrium dialysis experiments (KIRCHNER and FAULSTICH, 1986), using a tritium-labeled amatoxin, [^3H]-6'-*O*-methyl-dehydroxymethyl- α -amanitin. In 8 experiments the labeled amatoxin derivative exhibited a mean dissociation constant (K_D) of 4.0 ± 0.2 nM (graph not shown). In similar experiments the Fab fragment showed a K_D value of 3.8 nM, and was thus the same within the limits of error.

Toxicological studies

In NMRI mice used for the toxicological experiments, α -amanitin showed an LD_{50} value of 0.75 mg per kg body weight. The toxin was administered i.p., which gave the same LD_{50} value as that given by i.v. administration. The animals died from liver dystrophy, from the third day onwards.

When mice were poisoned i.p. with an LD_{50} of α -amanitin, and treated subsequently i.v. with an equivalent amount of monoclonal IgG, or Fab, all animals died, some of them earlier than the controls. This suggested to us that the antibody and the Fab had not decreased but rather enhanced the toxicity of α -amanitin. Indeed we showed that the LD_{50} of α -amanitin in the presence of its immunoglobulin was 0.39 mg per kg body weight, corresponding to a 2-fold increase of toxicity. A much greater increase of toxicity occurred when the poisoned mice were treated with Fab. In this case we determined an

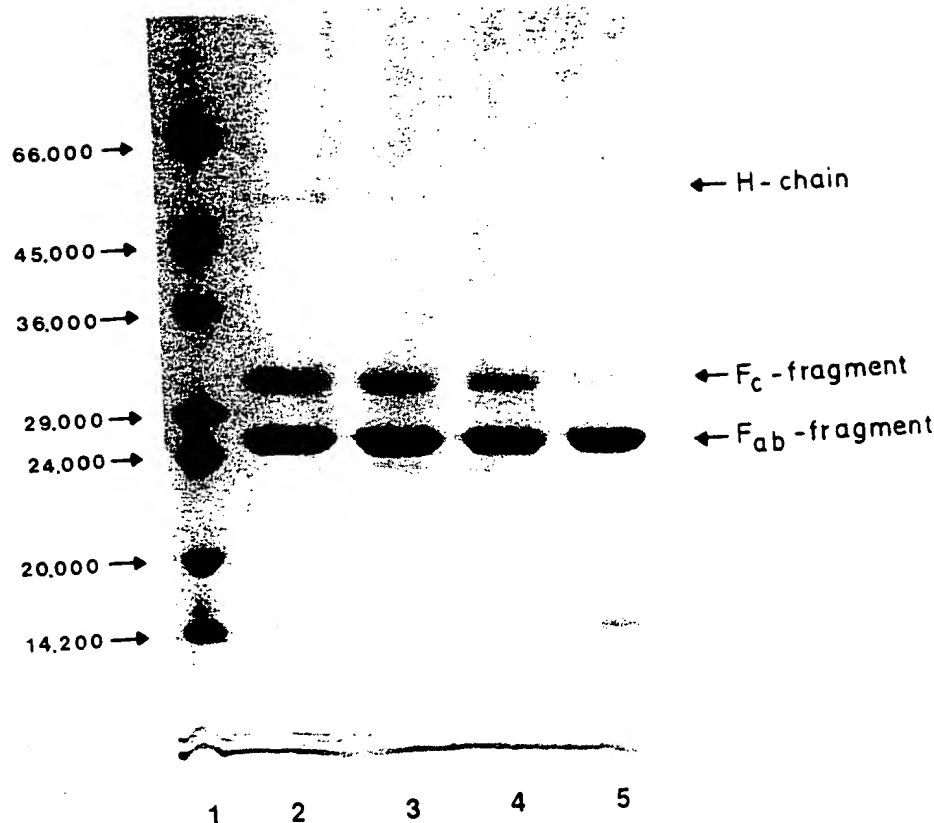


FIG. 2. MERCURIPAIN DIGESTION OF THE MONOCLONAL ANTIBODY AS MONITORED BY SDS-PAGE. Lane 2-5: reaction mixture after incubation with the enzyme for 1, 2, 3 and 4 hr. Lane 1: marker proteins: bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin inhibitor, 20,000; α -lactalbumin, 14,200.

LD₅₀ of 0.015 mg per kg body weight, which corresponds to a roughly 50-fold increase in toxicity (Table 1).

In order to identify the preferred target of α -amanitin under these conditions, livers and kidneys of the animals were examined by electron microscopy. In the Fab-treated mice the hepatocytes appeared completely normal (not shown), while considerable nuclear lesions were found in the proximal convoluted tubule cells of kidney (Fig. 3a, b). Since the nuclear lesions appeared as soon as 2 hr after poisoning we conclude that damage of the kidney tubule cells was the primary toxic event. According to this finding most of the animals are likely to have died from kidney failure, due to severe necrosis of the proximal convoluted tubules.

As with the Fab-treated mice, the animals treated with the whole immunoglobulin exhibited nuclear lesions predominantly in the kidney. These lesions occurred later than in the Fab-treated mice, but after 6 hr most of the nuclei of the proximal convoluted tubule cells had changed their structure. As in the Fab-treated animals the hepatocytes of the immunoglobulin treated mice appeared normal. There were, however, scattered nuclear lesions in some of the sinusoidal cells. After 48 hr the kidney cells of the immunoglobulin-treated mice had developed a severe necrosis.

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TABLE 1. SENSITIVITY OF NMRI MICE (FEMALE, 18-25 g) TO VARIOUS i.p. DOSES OF α -AMANITIN IN THE PRESENCE OR ABSENCE OF AMATOXIN-SPECIFIC IMMUNOGLOBULIN OR FAB-FRAGMENT

α			α /IgG2a			α /Fab		
Dose mg/kg	Mortality	Days of survival	Dose mg/kg	Mortality	Days of survival	Dose mg/kg	Mortality	Days of survival
1.00	8/8	3-4	0.59	6/6	3-16	0.80	4/4	1
0.81	12/13	3-4	0.51	4/4	2-4	0.40	6/6	1-5
0.76	3/6	3-4	0.49	6/6	2-5	0.20	4/4	3-5
0.59	3/20	3-4	0.29	0/4	-	0.10	5/5	4
0.51	0/10	-				0.05	4/4	4-5
						0.02	10/10	4-5
						0.01	2/9	4-5
						0.005	0/4	-

(α): α -amanitin. (α /IgG2a), (α /Fab): α -amanitin with subsequent i.v. administration of IgG2a (half molar amount) or Fab (molar amount).

Mortality = number of animals died/number of animals treated.

All protein fractions were in 0.9% NaCl and sterilized by ultrafiltration immediately before use.

In general, the monoclonal immunoglobulin as well as its Fab fragment had protected the hepatocytes of mice from amanitin toxicity, but had caused severe amanitin lesions in cells of the proximal convoluted tubules, leading, in both cases to an enhanced toxicity and to death by kidney failure.

DISCUSSION

Toxicity in mice of α -amanitin (i.p.), followed by i.v. administration of a monoclonal antibody, is very similar to the toxicity caused by i.v. administration of the amanitin-immunoglobulin complex, as indicated by the same LD₅₀ value and a similar course of intoxication (data not shown). Presumably the toxin when administered i.p. is trapped by the immunoprotein soon after entering blood circulation.

Trapping by immunoglobulins can decrease the filtration rate and thus retard renal excretion of small molecules as shown, for example, for digoxin (SCHMIDT *et al.*, 1974). This effect may enhance the toxicity of low mol. wt compounds, and it cannot be excluded that such an effect contributes to the higher toxic activity of α -amanitin in the presence of its immunoproteins. However, more important for the increased toxicity is certainly that complexing amatoxins to Fab or immunoglobulin changes their target cells.

Free amatoxins cause lesions predominantly in the parenchymal cells of the liver. In contrast, when amatoxins are covalently linked to albumin, or other proteins, they mainly affect cells involved in the protein turnover of the organism (DERENZINI *et al.*, 1974), such as macrophages, sinusoidal cells of the liver and protein-absorbing cells of kidney. Damage to these kinds of cells leads to markedly enhanced *in vivo* toxicity, (for a review see FAULSTICH and FIUME, 1985). As shown in the present study amatoxins, when administered together with their immunoproteins have an *in vivo* toxicity much higher than free amatoxins. In addition, they do not affect hepatocytes but those cells of liver and kidney which are largely involved in the turnover of proteins. We therefore conclude that the immunocomplexes of α -amanitin exhibit toxic activities very similar to amatoxins covalently linked to proteins.

While amatoxins conjugated with proteins mainly affect macrophages and sinusoidal cells, the immunocomplexes of amatoxins exhibit specificity for kidney cells. Nuclear lesions found in the cells of the proximal convoluted tubules are shown in Fig. 3. They



FIG. 3a. MOUSE PROXIMAL CONVOLUTED TUBULE CELL. 2 hr AFTER ADMINISTRATION OF 0.02 mg/kg BODY WEIGHT AMANITIN PLUS THE EQUI-MOLAR DOSE OF FAB.

The nucleus of the tubule cell shows a marked condensation and margination of chromatin and a clustering of ribonucleoprotein structures (arrows). BB, brush border. Bar, 1 μ m.

include fragmentation and segregation of ribonucleoprotein components, chromatin clumping, clustering of interchromatin-like granules and accumulation of perichromatin-like granules. These changes correspond to the lesions induced by free amatoxins in the hepatocytes of mouse and rat (MARINOZZI and FIUME, 1971).

The occurrence of nuclear lesions in kidney tubule cells indicates that complexes of immunoproteins and amatoxins are subject to glomerular filtration and tubular reabsorption. This is not self-evident, because the filtration rate of proteins decreases with increasing mol. wt. Albumin (66,000 mol. wt), for example, has a glomerular sieving coefficient of only 0.0001, (BALDAMUS *et al.*, 1971) and the filtration rate of immunoglobulins (155,000 mol. wt) is even lower (ROVIRA-HALBACH *et al.*, in press). Nevertheless filtration and reabsorption of the amanitin-immunoglobulin complex must be high enough to produce after several hr a toxin accumulation in the proximal convoluted tubule cells which causes kidney failure and finally death of the animals.

Fab fragments are filtered much more efficiently than immunoglobulins. With a mol. wt of 45,000 they resemble Bence-Jones proteins (44,000), which have a glomerular sieving coefficient of 0.09 (MAACK *et al.*, 1985). Accordingly, Fab fragments do not

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FIG. 3b. DETAIL OF FIG. 3a SHOWING THE CLUSTERED RIBONUCLEOPROTEIN STRUCTURES. F, fibrillar nucleolar fragments; G, nucleolar fragment with segregated fibrillar and granular components; IG, interchromatin granules. Bar, 0.2 μ m.

retard urinary excretion of small molecules as do immunoglobulins (BUTLER *et al.*, 1977). The high filtration rate of the Fab-amanitin-complex followed by extensive reabsorption in the tubules is clearly the crucial cause of the 50-fold increase of α -amanitin toxicity.

The present study was prompted by reports that high-affinity antibodies raised against digoxin were able to reverse the toxic effects of the drug in animals (CURD *et al.*, 1971; SCHMIDT and BUTLER, 1971; ZALGBERG *et al.*, 1983; SMITH *et al.*, 1979; BUTLER *et al.*, 1977). Moreover, Fab fragments of the digoxin-specific antibodies were successfully employed in a case of human suicidal digoxin poisoning (SMITH *et al.*, 1976). Such beneficial effects were not observed with the amatoxin-specific immunoproteins. This failure is most probably explained by the different locations of the targets of the two toxins. Incorporation of digoxin into tubule cells prevents the glycoside from binding to its target enzyme, the Na^+/K^+ -dependent ATPase located on the outer surface of plasma membranes. Alpha-amanitin, on the other hand, when complexed with its Fab, apparently uses the protein as a vehicle for penetration into tubule cells thereby gaining access to its target enzyme, the DNA-dependent RNA polymerase II (or B) (STIRPE and FIUME, 1967). Thus, the toxic activity of digoxin can be expected to be decreased, while that of α -amanitin may be greatly enhanced.

In conclusion, the present results speak against the feasibility of an immunotherapy of human *Amanita* poisoning using a monoclonal antibody or its Fab fragment. They confirm our previous finding that purified polyclonal antibodies of rabbit enhance amanitin toxicity in the mouse by a factor of two (KIRCHNER and FAULSTICH, 1986). On the other hand, the Fab-treated mice died from very low doses of α -amanitin indicative of a highly specific targeting of the toxin to the proximal convoluted kidney cells. It is

therefore possible that the α -amanitin/Fab-treated mouse could be employed as a model for studying acute kidney failure.

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